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Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing

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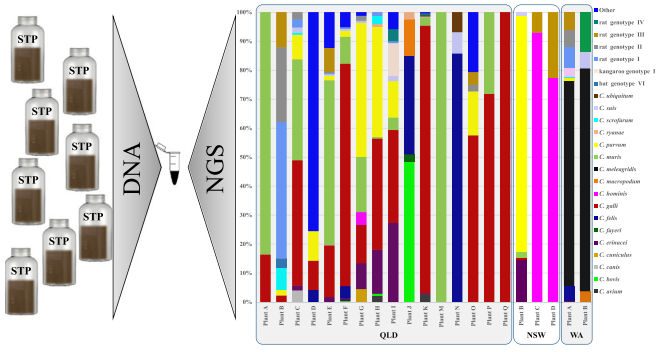
HIGHLIGHTS

- NGS analysis of *Cryptosporidium* spp. in raw wastewater samples ($n = 730$)
- Detection of large diversity of *Cryptosporidium* spp. and genotypes in wastewater
- Identification of *C. hominis*, *C. parvum* and *C. meleagridis* in untreated wastewater
- Potential contribution of livestock, wildlife and birds to wastewater contamination

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater recycling is an increasingly popular option in worldwide to reduce pressure on water supplies due to population growth and climate change. *Cryptosporidium* spp. are among the most common parasites found in wastewater and understanding the prevalence of human-infectious species is essential for accurate quantitative microbial risk assessment (QMRA) and cost-effective management of wastewater. The present study conducted next generation sequencing (NGS) to determine the prevalence and diversity of *Cryptosporidium* species in 730 raw influent samples from 25 Australian wastewater treatment plants (WWTPs) across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA), between 2014 and 2015. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using quantitative PCR (qPCR), oocyst numbers were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR, and positives were characterized using NGS of 18S amplicons. Positives were also screened using *C. parvum* and *C. hominis* specific qPCRs. The overall *Cryptosporidium* prevalence was 11.4% (83/730): 14.3% (3/21) in NSW; 10.8% (51/470) in QLD; and 12.1% (29/239) in WA. A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. In NSW, *C. hominis* and *Cryptosporidium* rat genotype III were the most prevalent species (9.5% each). In QLD, *C. galli*, *C. muris and C. parvum* were the three most prevalent species (7.7%, 5.7%, and 4.5%,

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respectively), while in WA, *C. meleagridis* was the most prevalent species (6.3%). The oocyst load/Litre ranged from 70 to 18,055 oocysts/L (overall mean of 3426 oocysts/L: 4746 oocysts/L in NSW; 3578 oocysts/L in QLD; and 3292 oocysts/L in WA). NGS-based profiling demonstrated that *Cryptosporidium* is prevalent in the raw influent across Australia and revealed a large diversity of *Cryptosporidium* species and genotypes, which indicates the potential contribution of livestock, wildlife and birds to wastewater contamination.

1. Introduction

Australia is the driest of the world's inhabited continents, with the lowest percentage of rainfall as run-off and the lowest amount of water in rivers (Anonymous, 2004). Drinking water resources are under considerable strain as a result of major shifts in long-term climate change, and climate predictions for all Australian States and Territories suggest increasing temperatures, a decline in average rainfall, but increasing severity and frequency of storm events (Garnaut Review, 2008). Consequently, there is increasing pressure for more efficient use of water resources, both in urban and rural environments (Toze, 2006a). Recycling wastewater will help address these challenges and is a prominent option among the various alternative sources of water in both developing and developed countries (Miller, 2006; Mekala and Davidson, 2016). However, infection with pathogenic microorganisms is a major risk factor (Rodriguez-Manzano et al., 2012) and therefore water destined for reuse must be fit for purpose (Toze, 2006b).

The waterborne parasite *Cryptosporidium* represents an important public health concern for water utilities, as it is a major cause of diarrhoea and there is neither a vaccine nor an effective treatment (Ryan et al., 2016; Zahedi et al., 2016a). *Cryptosporidium* is particularly suited to waterborne transmission as the oocyst stage is highly resistant to chlorine disinfection and can penetrate and survive routine water and wastewater treatment systems (King and Monis, 2007; King et al., 2017; Ryan et al., 2017a). The parasite has been responsible for numerous large-scale waterborne outbreaks worldwide (Efstratiou et al., 2017) and is highly prevalent in wastewater (Amorós et al., 2016; Ma et al., 2016).

Cryptosporidium species are currently monitored in wastewater using standard detection methodologies (i.e. fluorescence microscopy using EPA method 1623 - USEPA, 2012), however, this method cannot discriminate between different *Cryptosporidium* species. Of the 37 recognised *Cryptosporidium* species, *C. hominis* and *C. parvum* are the dominant species that infect humans (Ryan et al., 2016; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018). As not all species of *Cryptosporidium* are infectious to humans (Ryan et al., 2016), understanding the diversity of *Cryptosporidium* in wastewater is crucial for more accurate quantitative microbial risk assessment (QMRA), for proper management of wastewater and its recycling. Due to the complex composition, abundance, and distribution patterns of *Cryptosporidium* species present in wastewater samples, molecular techniques such as conventional PCR and Sanger sequencing-based genotyping methods are unable to resolve complex DNA mixtures due to mixed sequencing chromatograms and are also unable to detect low abundance species or variants of *Cryptosporidium* (which typically appear as a "bumpy baseline" in Sanger chromatograms) (Murray et al., 2015; Paparini et al., 2015; Grinberg and Widmer, 2016).

Next-generation sequencing (NGS) technologies have allowed the comprehensive characterization and deep coverage of microbial community structure and diversity in environmental samples such as soil, water, the atmosphere and other environments (Cruaud et al., 2014). NGS is also more sensitive for the detection of less abundant species within microbial communities (Salipante et al., 2013). Recently, NGS approaches have been described that examine the composition and diversity of microbial communities (Shanks et al., 2013; Ma et al., 2015; Newton et al., 2015; Xu et al., 2017), adenovirus (Ogorzaly et al., 2015), norovirus (Prevost et al., 2015), astrovirus (Brinkman et al., 2013) and protists (Maritz et al., 2017) in sewage. However, to the best of the authors' knowledge, to date no large scale longitudinal studies have been undertaken to investigate the composition and diversity of *Cryptosporidium* species in wastewater using high-throughput amplicon NGS. As the costs of NGS continue to decrease and the bioinformatics analysis of data continues to improve, NGS screening of wastewater samples has become more feasible (Muir et al., 2016).

Therefore, the aim of the present study was to use NGS, for the first time on a large scale, to more accurately determine the prevalence and composition of *Cryptosporidium* species in Australian WWTPs across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

2. Materials and methods

2.1. Study sites and sample collection

In NSW, WWTP samples (250 mL raw influent) were collected on a monthly interval over five months (April 2015 to August 2015). A total of 21 WWTP samples were collected from four wastewater plants within the WaterNSW area of operations (greater Sydney) (Table 1). In QLD, a total of 470 WWTP samples (250 mL raw influent) were collected on fortnightly intervals from WWTP sites ($n = 19$) across south east Queensland (Table 1) over a year (January 2014 to January 2015). In WA, a total of 239 WWTP samples (250 mL raw influent) were collected from two treatment plants on weekly intervals from December 2014 to December 2015 (Table 1). All raw influent WWTP samples were collected into individual 250 mL collection pots and stored at 4 °C until required and samples collected in NSW and QLD were shipped to Murdoch University for analysis.

2.2. Sample processing and DNA isolation

All 250 mL WWTP samples were transferred to 50 mL centrifuge tubes, and evenly weighed tubes ($n = 5$) were prepared from the same samples. These samples were centrifuged at 10,000 ×*g* for 20 min and pellets from the same samples were mixed together again. DNA was extracted from aseptically separated 250 mg aliquots of each sample (pellet), using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA) (Walden et al., 2017). An extraction blank (no WWTP sample) and a positive extraction control (a *Cryptosporidium* positive faecal sample from a kangaroo), was included in each extraction batch, as a process control for extraction efficiency. Purified DNA was stored at −20 °C prior to molecular analyses.

2.3. qPCR and oocyst enumeration

All WWTP sample extracts were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014). A spike analysis of the 18S qPCR assay (addition of 0.5 μL of positive control DNA into test samples) was conducted on randomly selected negative samples from each group of DNA extractions, to determine if negative results were due to PCR inhibition by comparing the cycle threshold (Ct) values of the spike and the positive control (both with same

concentration of DNA). In addition, *Cryptosporidium* oocyst concentrations in each sample (oocyst numbers per litre) were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR (ddPCR) (QX100™ droplet digital PCR system, Bio-Rad), which has the advantage of providing more accurate quantitation (Yang et al., 2014). Briefly, target copy numbers of the 18S gene detected in individual samples were converted to estimates of oocyst numbers based on the fact that the 18S gene in *Cryptosporidium* has five copies per haploid sporozoite (Le Blancq et al., 1997; Abrahamsen et al., 2004), and there are four haploid sporozoites per oocyst. Therefore, every 20 copies of 18S detected by qPCR were equivalent to one oocyst. To estimate oocyst density per litre, oocyst numbers detected per 250 mg aliquots of each sample (pellet) were extrapolated to the corresponding total pellet weight extracted from each 250 mL wastewater sample, and then multiplied by four.

2.4. Next generation sequencing

Samples that were positive by qPCR were analysed by NGS on the MiSeq (Illumina) platform at the 18S locus using the 18S iF/iR primers (Morgan et al., 1997) that were modified to contain MiSeq adapter sequences on the 5′ and 3′ end as previously described (Paparini et al., 2015). The library was prepared as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) with the following modifications: all PCR amplicons (uniquely indexed per sample) were double purified using the Agencourt AMPure XP Bead PCR purification protocol (Beckman Coulter Genomics, USA) and pooled in approximate equimolar ratios (based on gel electrophoresis). Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads) following the manufacturer's recommendations. Two no-template controls and two DNA extraction reagent blank controls were included in the library preparation and distributed between samples in the PCR plate layout. All no-template and extraction reagent blank controls produced no detectable amplification of *Cryptosporidium* DNA throughout the library preparation. This indicated that level of cross contamination between samples, or from the laboratory environment, was below the detection limit of the library preparation procedure and for this reason were not sequenced. We have also previously sequenced extraction blanks and no-template controls from other NGS studies in our laboratory, and after quality filtering, <10 reads were detected in those samples.

2.5. Species-specific PCR for detection and enumeration of C. hominis and C. parvum

All WWTP samples positive for *Cryptosporidium* spp. by qPCR at the 18S locus were also screened and enumerated independently using primers and species-specific minor groove binder (MGB) probes to a unique *Cryptosporidium* specific gene (Clec) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain to confirm the presence/absence of *C. hominis* and *C. parvum* as described by Yang et al. (2013).

2.6. Statistical analysis

The overall prevalence of *Cryptosporidium* in samples collected from each WWTP was expressed as the percentage of samples positive by combined qPCR and NGS, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocyst equivalents measured by ddPCR. Odds ratios (OR) and their 95% confidence intervals were used to measure the strength of association of season (risk factor) with the occurrence of the *Cryptosporidium* species in WWTP samples. Chi-square and non-parametric analyses were performed using IBM SPSS 21.0 (statistical package for the social sciences) for Windows (SPSS Inc. Chicago, USA) to determine if there were any associations between the prevalence and concentration of *Cryptosporidium* oocysts at different sampling seasons and across states.

2.7. Bioinformatics analysis

Illumina MiSeq sequencing resulted in 1,068,270,250 bp paired-end reads with 78% of the basecalls >Q30. Paired-end reads were merged and quality filtered with USEARCH v9.2 (Edgar, 2010), retaining reads with >50 bp merged overlap, <0.1% expected error, no mismatches in the primer sequences, a minimum length of 200 bp, and a minimum of 100 identical replicate copies as previously described (Zahedi et al., 2017b). Primer sequences and any distal bases were also removed from all reads. Reads were then denoised and chimera filtered with the UNOISE3 algorithm (Edgar, 2016) to generate 169 zero-radius operational taxonomic units (ZOTUs) that represent unique biologically correct sequences (Edgar, 2016). *Cryptosporidium* 18S ZOTU sequences were assigned taxonomy by comparing ZOTUs to a curated custom database containing 63 reliable 18S reference sequences from 35 *Cryptosporidium* species and 28 genotypes extracted from GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Taxonomy was only assigned if there was a single unambiguous BLAST hit with >99% pairwise identity over >98% of the query ZOTU sequence. Of the 169 ZOTUs generated, 62 did not match any known *Cryptosporidium* species or genotypes. These non-specific ZOTUs were compared to GenBank using BLAST, and where possible, taxonomy was assigned when queries hit reference sequences with >99% identity over >98% of the query reads and matched to no other taxa at the same level. Many of these non-specific ZOTUs were assigned to uncultured eukaryotes or benign waterborne fungi, algae and dinoflagellates (Supplementary Table 1). Although abundant at the ZOTU level, these non-specific sequences represented a very small proportion of the total reads per sample (mean 0.71%).

3. Results

3.1. Overall prevalence of Cryptosporidium in WWTP samples

In the present study, a total of 730 WWTP samples from 25 WWTPs across three states in Australia (NSW, QLD and WA) were screened using qPCR, and the composition of *Cryptosporidium* species in positive samples was determined by NGS. Results were tabulated as the prevalence of the most abundant single species (determined by NGS), detected per sample (Table 2) and the prevalence of all *Cryptosporidium* species detected across all samples regardless of their abundance (Table 3). Overall, *Cryptosporidium* was detected in 11.4% (83/730; 95% CI, 9.2–13.9) of WWTP samples collected across three states. (Table 4 and Supplementary Table 1). This comprises a prevalence of 14.3% (3/21; 95% CI, 3–36.3) in NSW, 10.8% (51/470; 95% CI, 8.2–14) in QLD and 12.1% (29/239; 95% CI, 8.3–17) in WA. However, there was no significant difference between the prevalence in different states ($p > 0.05$). In general, across the three states, samples collected in summer were 1.9 times more likely to have *Cryptosporidium* than samples collected during winter months (Odds ratio = 1.9; 95% CI, 1.2–3.4), but there was no statistical difference between samples collected in spring, winter and autumn ($p > 0.05$). For NSW, samples were only collected for autumn and winter.

In QLD, the prevalence of *Cryptosporidium* peaked at 17.5% (18/103; 95% CI, 10.7–26.2) during summer months (averaged over two partial summers; 2014 and 2015), when the samples were 2.3 times more likely to have *Cryptosporidium* than samples collected during winter months (averaged over winter 2014 and 2015) (Odds ratio = 2.3; 95% CI, 1.2–5.2). There was no significant difference between the prevalence in spring, autumn and winter ($p > 0.05$). Unlike QLD, WA had the highest prevalence of *Cryptosporidium* in WWTP samples collected

Table 1
Wastewater treatment plants (WWTPs) included in the present study.

	Type of plant	Rural/urban plant	Source of sewage	Size of community served	Trade waste received/not received?	Nature of trade waste	Any storm water intrusion	Animal presence/activity around the plant
QLD								
Plant A	Trickle filter	Rural	Domestic/some commercial	9000	Not received	NA	Yes	Yes (cattle in neighbouring properties)
Plant B	Lagoon	Rural	Domestic/some commercial	500	Not received	NA	Yes	Yes (cattle in neighbouring field/wildlife, kangaroo/koalas)
Plant C	Extended activated sludge	Rural	Domestic/some commercial	1000	Not received	NA	Yes	Yes (bats)
Plant D	Activated sludge	Rural	Principally domestic	36,000	Small portion is trade waste	Industrial trade waste	Yes	No
Plant E	Activated sludge	Urban	Principally domestic	45,000	Significant portion is trade waste	Chemical trade waste/tannery waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant F	Activated sludge	Rural	Principally domestic	1000	Small portion is trade waste	Restaurant waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant G	Activated sludge	Rural	Principally domestic	105,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant H	Activated sludge	Rural	Principally domestic	12,500	Insignificant portion is trade waste	Rendering plant that pre-treats waste before sending waste to WWTP	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant I	Activated sludge	Rural	Principally domestic	2400	Insignificant portion is trade waste	Industrial waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant J	Activated sludge	Urban	Principally domestic	118,000	Significant portion is trade waste	Restaurant waste	Yes	No
Plant K	Activated sludge	Urban	Principally domestic	60,000	Small portion is trade waste	Seafood waste	Yes	No
Plant L	Activated sludge	Rural	Principally domestic	126,000	Significant portion is trade waste	Industrial trade waste	Yes	No
Plant M	Activated sludge	Rural	Decommissioned (August 2014)	Decommissioned	Decommissioned	NA	Yes	N/A
Plant N	Activated sludge	Rural	Principally domestic	22,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant O	Facultative lagoons	Rural	Principally domestic	300	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with

								possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant P	Activated sludge	Rural	Principally domestic	43,000	Significant portion is trade waste	Food manufacturing waste	Yes	No
Plant Q	Activated sludge	Rural	Principally domestic	47,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant R	Activated sludge	Urban	Principally domestic	5000	Small portion is trade waste	Restaurant waste	Yes	No
Plant S	Activated sludge	Rural	Principally domestic	26,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
NSW								
Plant A	Oxidation ditch (Pasveer), sludge lagoons	Urban	Domestic and industrial	2000	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
Plant B	IDEA tank, oxidation ditch (Pasveer) (not in operation), sludge drying/lagoons	Urban	Domestic and industrial	5400	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (Native wildlife (wombats, kangaroos and birds))
Plant C	IDEA tank, sludge drying/lagoons.	Urban	Domestic and industrial	9000	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
Plant D	IDAL aeration, oxidation ditch (Pasveer), sludge drying/lagoons	Urban	Domestic and industrial	14,600	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
WA								
Plant A	Activated sludge	Urban	Principally domestic (but also receives industrial waste through the sewer network)	75,000	Received	Septage and grease trap waste, abattoir and farm waste (through third party tankers)	Yes	Limited (foxes, feral cats, birds and snakes)
Plant B	Pond system	Urban	Domestic	5000	Not received	NA	Yes	Cattle, kangaroos and birds (turtles snakes and birds live in the ponds)

NA = Not Available.
IDEA = Intermittently Decanted Extended Aeration.
IDAL = Intermittently Decanted Aerated Lagoons.

Table 2
Prevalence of the most abundant *Cryptosporidium* species detected by NGS of individual wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (based on a single species that was the most abundant species detected in each sample).

Host	*C. parvum* No +/total no (% proportion + 95% CI)	*C. hominis* No +/total no (% proportion + 95% CI)	*C. bovis* No +/total no (% proportion + 95% CI)	*C. muris* No +/total no (% proportion + 95% CI)	*C. erinacei* No +/total no (% proportion + 95% CI)	*C. meleagridis* No +/total no (% proportion + 95% CI)	*C. galli* No +/total no (% proportion + 95% CI)	*C. canis* No +/total no (% proportion + 95% CI)	*C. felis* No +/total no (% proportion + 95% CI)	*C. suis* No +/total no (% proportion + 95% CI)	*C. macropodum* No +/total no (% proportion + 95% CI)	Other No +/total no (% proportion + 95% CI)
QLD												
Plant A	ND	ND	ND	1/25 (4%, 0.1–20.4)	ND	ND	1/25 (4%, 0.1–20.4)	ND	ND	ND	ND	ND
Plant B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2[a]/20 (10%, 1.2–31.7)
Plant C	ND	ND	ND	ND	ND	ND	3/26 (11.5%, 2.4–30.2)	1/26 (3.8%, 0.1–19.6)	ND	ND	ND	ND
Plant D	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND
Plant E	ND	ND	ND	2/41 (4.9%, 0.6–16.5)	1/41 (2.4%, 0.1–12.9)	ND	7/41 (17%, 7.2–32.1)	ND	ND	ND	ND	ND
Plant F	ND	ND	1/40 (2.5%, 0.1–13.2)	1/40 (2.5%, 0.1–13.2)	ND	ND	4/40 (10%, 2.8–23.7)	ND	1/40 (2.5%, 0.1–13.2)	ND	ND	ND
Plant G	2/41 (4.9%, 0.6–16.5)	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	2/41 (4.9%, 0.6–16.5)	ND	ND	ND	ND	ND
Plant H	1/41 (2.4%- 0.1–12.9)	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	2/41 (4.9%, 0.6–16.5)	ND	ND	ND	ND	ND
Plant I	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND
Plant J	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND	ND	ND	3/41 (7.3%, 1.5–19.9)	ND
Plant K	ND	ND	ND	ND	ND	ND	5/41 (12.2%, 4.1–26.2)	ND	ND	ND	ND	ND
Plant L	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant M	ND	ND	ND	1/7 (14.3%, 0.4–57.9)	ND	ND	ND	ND	ND	ND	ND	ND
Plant N	ND	ND	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND
Plant O	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant P	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant Q	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	4/123 (3.2%, 0.9–8.1)	ND	2/81 (2.5%, 0.3–8.6)	6/154 (3.9, 1.4–8.3)	2/81 (2.5%, 0.3–8.6)	ND	28/308 (9.1%, 6.1–12.9)	1/26 (3.8%, 0.1–19.6)	3/85 (3.5%, 0.7–10)	ND	3/41 (7.3%, 1.5–19.9)	2/20 (10%, 1.2–31.7)
NSW												
Plant A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant B	1/5 (20%, 0.5–71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant C	ND	1/5 (20%, 0.5–71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant D	ND	1/6 (16.7%, 0.4–64.1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	1/5 (20%, 0.5–71.6)	2/11 (18.2%, 2.3–51.8)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
WA												
Plant A	3/146 (2.1%, 0.4–5.9)	ND	ND	ND	ND	7/146 (4.8%, 1.9–9.6)	ND	ND	5/146 (3.4%, 1.1–7.8)	ND	ND	6[b]/146 (4.1%, 1.5–8.7)
Plant B	ND	ND	ND	ND	ND	6/93 (6.5%, 2.4–13.5)	ND	ND	ND	1/93 (1.1%, 0–5.8)	1/93 (1.1%, 0–5.8)	3[c]/93 (3.2%, 0.7–9.1)
Total	3/146 (2.1%, 0.4–5.9)	ND	ND	ND	ND	13/239 (5.4%, 2.9–9.1)	ND	ND	5/146 (3.4%, 1.1–7.8)	1/93 (1.1%, 0–5.8)	1/93 (1.1%, 0–5.8)	9/239 (3.8%, 1.7–7)

ND = not detected.
[a] Rat genotype I ($n = 1$), rat genotype II ($n = 1$).
[b] Rat genotype I ($n = 2$), rat genotype II ($n = 2$), rat genotype III ($n = 1$), kangaroo genotype I ($n = 1$).
[c] Kangaroo genotype I ($n = 3$).

Table 3
Prevalence of all *Cryptosporidium* species/genotypes detected by NGS in wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (regardless of abundance).

Cryptosporidium spp.	No+/total no (% proportion + 95% CI)		
	NSW	QLD	WA
C. hominis	**2/21 (9.5%; 95% CI, 1.2–30.4)**	**2/470 (0.4%; 95% CI, 0.1–1.5)**	**ND**
C. parvum	**1/21 (4.8%; 95% CI, 0.1–23.8)**	**21/470 (4.5%; 95% CI, 2.8–6.7)**	**3/239 (1.3%; 95% CI, 0.3–3.6)**
C. avium	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND
C. bovis	ND	5/470 (1.1%; 95% CI, 0.3–2.5)	ND
C. canis	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. cuniculus	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. erinacei	1/21 (4.8%; 95% CI, 0.1–23.8)	14/470 (3.0%; 95% CI, 1.6–4.9)	3/239 (1.3%; 95% CI, 0.3–3.6)
C. fayeri	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. felis	ND	4/470 (0.8%; 95% CI, 0.2–2.2)	5/239 (2.1%; 95% CI, 0.7–4.8)
C. galli	1/21 (4.8%; 95% CI, 0.1–23.8)	36/470 (7.7%; 95% CI, 5.4–10.4)	ND
C. macropodum	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	1/239 (0.4%; 95% CI, 0–2.3)
C. meleagridis	ND	ND	14/239 (5.9%; 95% CI, 3.2–9.6)
C. muris	1/21 (4.8%; 95% CI, 0.1–23.8)	27/470 (5.7%; 95% CI, 3.8–8.2)	1/239 (0.4%; 95% CI, 0–2.3)
C. ryanae	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	ND
C. scrofarum	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	2/239 (0.8%; 95% CI, 0.1–3)
C. suis	1/21 (4.8%; 95% CI, 0.1–23.8)	11/470 (2.3%; 95% CI, 1.2–4.1)	1/239 (0.4%; 95% CI, 0–2.3)
C. ubiquitum	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND
Bat genotype VI	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
Kangaroo genotype I	ND	1/470 (0.2%; 95% CI, 0–1.2)	4/239 (1.7%; 95% CI, 0.5–4.2)
Rat genotype I	ND	9/470 (1.9%; 95% CI, 0.91–3.6)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype II	ND	5/470 (1.1%; 95% CI, 0.3–2.5)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype III	2/21 (9.5%; 95% CI, 1.2–30.4)	4/470 (0.8%; 95% CI, 0.2–2.2)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype IV	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND

ND = not detected.
The bold data indicates the most two common species of *Cryptosporidium* reported in humans in Australia, accounting for >95% of human infections.

during spring (16.8%; 95% CI, 8.3–28.5), while there was no significant difference between the prevalence in summer, autumn and winter ($p > 0.05$). Although the prevalence of different species peaked at different times (Supplementary Table 1), in WA (Plant A), there was a winter peak in both *C. parvum* and rate genotype 1 and a spring peak for *C. felis*, and in plant B, there was a summer peak for *C. suis* (Supplementary Table 1).

3.2. Prevalence of all Cryptosporidium species/genotypes as determined by NGS (regardless of abundance)

A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS (Table 3, and Supplementary Table 1). *Cryptosporidium hominis* and rat genotype III were the most prevalent species detected in wastewater samples collected from NSW (9.5% each, 2/21; 95% CI, 1.2–30.4). In addition to *C. hominis* and rat genotype III, *C. parvum* was detected in one NSW sample only (4.8%, 1/21; 95% CI; 0.1–23.8) and *C. erinacei*, *C. galli*, *C. muris* and *C. suis* were also detected in the same sample in low abundance (Table 3 and Supplementary Table 1). In QLD, of the 51 WWTP samples positive for *Cryptosporidium*, NGS detected more than one *Cryptosporidium* species/genotype in 42 samples, ranging from two to eight species in individual samples, whereas in nine samples, only one *Cryptosporidium* species/genotype was identified. In general, the prevalence of different *Cryptosporidium* species/genotypes detected by NGS in WWTP samples across QLD ranged from 0.2% to 7.7% (Table 3). *Cryptosporidium galli* (7.7%), *C. muris* (5.7%) and *C. parvum* (4.5%) were the three most prevalent (and abundant) species detected in WWTP samples from QLD, followed by *C. erinacei* (3.0%), *C. suis* (2.3%) and rat genotype I (1.9%) (Table 3), and were significantly more prevalent than all other species detected in samples from QLD ($p < 0.05$) (Table 3). Unlike QLD, the majority of samples positive for *Cryptosporidium* in WA (22/29) contained only one species/genotype of *Cryptosporidium* (75.9%; 95% CI, 56.5–89.7), and only seven samples were identified with mixed *Cryptosporidium* species present (24.1%; 95% CI, 10.3–43.5). *Cryptosporidium meleagridis* was detected in 5.9% (15/239; 95% CI, 3.2–9.6) of wastewater samples collected from WA, and was significantly more prevalent than any other species detected ($p < 0.05$). However, there was no significant difference between the prevalence of other *Cryptosporidium* species detected in WA samples ($p > 0.05$). *Cryptosporidium C. parvum* (1.3%), *C. erinacei* (1.3%), *C. scrofarum* (0.8%) and *C. muris* (0.4%) were detected at a low prevalence only in samples with mixed *Cryptosporidium* species/genotypes in WA (Table 3 and Supplementary Table 1).

3.3. Abundance and diversity of all Cryptosporidium reads determined by NGS

Overall, the highest number of reads (sequences) across the 83 WWTP samples positive for *Cryptosporidium* was assigned to *C. galli* (22.8% of all sequences analysed). This was followed by *C. meleagridis* (15.7%), *C. muris* (11.9%), *C. felis* (8.7%), *C. parvum* (6.8%), kangaroo genotype I (JF316651) (4.9%), *C. macropodum* (4.5%), rat genotype I (3.9%), rat genotype II (2.9%), *C. hominis* (2.5%), *C. erinacei* (2.5%), rat genotype III (2.1%), *C. suis* (1.7%), *C. bovis* (0.8%), *C. scrofarum* (0.5%), *C. canis* (0.4%), *C. fayeri* (0.3%), *C. cuniculus* (0.2%), *C. avium* (0.2%), *C. ubiquitum* (0.1%), *C. ryanae* (0.1%), rat genotype IV (0.1%) and bat genotype VI (0.1%) (Supplementary Table 1 and Fig. 1). There were also a small proportion of NGS sequences (6.1%), across 11 samples, that were not assigned to any *Cryptosporidium* species or genotypes (Supplementary Table 1 and Fig. 1). In general, at the individual sample level across the three states, the number of species identified in individual wastewater samples ranged from one to eight species.

3.4. Additional confirmation of presence/absence and enumeration of C. hominis and C. parvum in WWTP samples using a species-specific qPCR

Of 83 WWTP samples positive for *Cryptosporidium* spp. NGS detected *C. parvum* and *C. hominis* in 25 and four samples, respectively, including two samples that contained both species (QLD-E76 and QLD-G115) (Table 3, Table 5 and Supplementary Table 1). A *C. parvum* species-specific qPCR assay confirmed the presence of *C. parvum* in 20/25 samples, but failed to amplify the remaining five samples, which were previously identified by NGS to contain *C. parvum* sequences in low abundance, ranging from 113 to 535 reads (Table 5). The occurrence of *C. hominis* in 3/4 WWTP samples was also confirmed a *C. hominis*-

Table 4
Seasonal prevalence, the mean and median *Cryptosporidium* oocyst concentration in positive samples per litre (mean, median with range in parenthesis (determined by qPCR) per season across three states of Australia; NSW, QLD and WA).

State	Summer		Autumn		Winter		Spring		Overall	
	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range
QLD	18/103 (17.5%, 10.7–26.2)	5966, 3821 (192–18,055)	11/105 (10.5%, 5.3–18)	2583, 1974 (203–8134)	10/131 (7.6%, 3.7–13.6)	2323, 1131 (172–14,602)	12/131 (9.2%, 4.8–15.5)	1953, 1107 (70–6301)	51/470 (10.8%, 8.2–14)	3578, 1619 (70–18,055)
NSW	NC	NC	1/17 (5.9%, 0.1–28.7)	1428, NA (only one sample)	2/4 (50%, 6.8–93.2)	6405, 6405 (two samples)	NC	NC	3/21 (14.3%, 3–36.3)	4746, 4373 (1428-8438)
WA	9/60 (15%, 7.1–26.6)	1632, 1590 (327–2842)	4/60 (6.8%, 1.8–16.2)	1217, 936 (599–2398)	6/59 (10.2%, 3.8–20.8)	2107, 1928 (1105-3326)	10/60 (16.8%, 8.3–28.5)	6326, 3805 (2267-16,812)	29/239 (12.1%, 8.3–17)	3292, 2398 (327–16,812)
Overall	27/163 (16.6%, 11.2–23.2)	4521, 2191 (192–18,055)	16/174 (9.2%, 5.3–14.5)	2170, 1260 (203–8134)	18/202 (8.9%, 5.4–13.7)	2704, 1627 (172–14,602)	22/191 (11.5%, 7.4–16.9)	3941, 2823 (70–16,812)	83/730 (11.4%, 9.2–13.9)	3426, 1828 (70–18,055)

NC = not collected.

specific qPCR, with no *C. hominis* amplification in a single sample which was previously confirmed by NGS to contain *C. hominis* in low abundance (QLD-E76) (Table 5 and Supplementary Table 1). The concentration of *C. hominis* and *C. parvum* oocysts per litre in these samples ranged from 386 to 3294 and from 14 to 6314, respectively (Table 5). The absence of *C. hominis* and *C. parvum* in the remaining samples ($n = 56$) was confirmed by the *C. hominis* and *C. parvum* species-specific qPCR assays (Table 5).

3.5. Enumeration of Cryptosporidium oocysts in wastewater samples using qPCR

Cryptosporidium oocyst concentration per litre was estimated using qPCR standards calibrated by ddPCR at the 18S locus (Table 4). Overall, the oocyst load per litre in samples collected across the three states ranged from 70 to 18,055 oocysts/L and the mean was 3426 oocysts/L (Table 4). The mean *Cryptosporidium* oocyst concentration in samples collected from WWTPs in NSW was the highest among the states (4746 oocysts/L). However, due to the low number of samples collected from NSW ($n = 21$), compared to 470 from QLD and 239 from WA, statistical analysis of oocyst load was only conducted for QLD and WA to avoid potential bias in the analysis.

The mean number of oocysts per litre in samples collected from the two WWTPs in WA over four seasons was 3292 oocysts/L (ranging from 327 to 16,812), while the mean *Cryptosporidium* oocyst concentration in these samples peaked during spring 2015 at 6326 oocysts/L (ranging from 2267 to 16,812). This corresponded with a peak of prevalence at this time (spring 2015) (16.8%; 95% CI, 8.3–28.5) (Table 4).

Compared to WA, the overall mean *Cryptosporidium* oocyst concentration in WWTP samples from QLD was relatively higher (3578 oocysts/L, ranging from 70 to 18,055). Seasonal mean concentrations (averaged over the two same seasons in 2014 and 2015) were 1953 oocysts/L in spring, 2323 oocysts/L in winter, 2583 oocysts/L in autumn and 5966 oocysts/L in summer. This also corresponded with a peak prevalence of 17.5% (95% CI, 10.7–26.2) during summer (averaged over summer 2014, 2015 and 2016) (Table 4).

4. Discussion

The present study has demonstrated the utility of NGS in detecting mixtures of *Cryptosporidium* species and genotypes in sewage and has shown that they are frequently present but variable and diverse in space, time and composition. The overall prevalence of *Cryptosporidium* in WWTP samples across Australia was 11.4% (83/730). Previous studies have reported prevalence ranging from 6.4% to 100% (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003; Hanninen et al., 2005; Cantusio Neto et al., 2006; Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Ottoson et al., 2006; Robertson et al., 2006; Castro-Hermida et al., 2008; Feng et al., 2009; Dungeni and Momba, 2010; Liu et al., 2011; Ajonina et al., 2012; Ben Ayed et al., 2012; Li et al., 2012; Gallas-Lindemann et al., 2013, 2016; Hachich et al., 2013; Spanakos et al., 2015; Amorós et al., 2016; Hatam-Nahavandi et al., 2016; Ulloa-Stanojlović et al., 2016; ; Huang et al., 2017; Imre et al., 2017; Ramo et al., 2017; Santos and Daniel, 2017). However, to the best of the authors' knowledge, in Australia little published information is available on the prevalence and composition of *Cryptosporidium* species in wastewater (King et al., 2015, 2017).

In the present study, a total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. This is higher than the diversity reported in previous studies due to the ability of NGS to detect mixtures of sequences in low abundance. Wastewater treatment networks however, rarely contain only domestic wastewater; they often also contain wastewater from industrial sources and can be influenced by environmental water sources, such as stormwater or groundwater (Pandey et al., 2014). In addition, wild animals may directly contribute to contamination of sewage, such as rodents in the sewer networks or birds

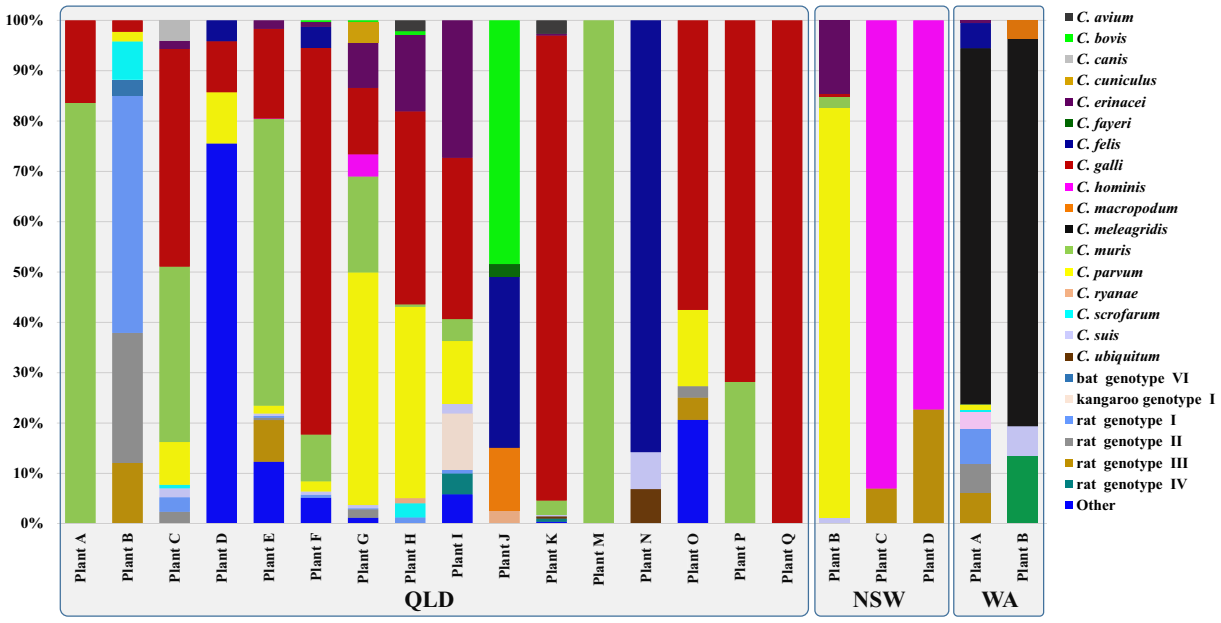

Fig. 1. Percent composition of 18S sequences from *Cryptosporidium* species detected in wastewater treatment plant samples from NSW, QLD and WA.

present at wastewater treatment plants. Therefore, the presence of a variety of *Cryptosporidium* species from livestock, wildlife and birds in sewage samples may be attributed to other sources such as stormwater or industrial waste from animal processing.

In the present study, of 83 WWTP samples positive for *Cryptosporidium* spp., NGS detected *C. hominis* and *C. parvum* in only 27 samples (32.5%; 95% CI, 22.6–43.7), of which two samples contained both species. A *C. hominis* and *C. parvum* species-specific qPCR provided further support for the lack of *C. hominis* and *C. parvum* in the majority of samples, although it failed to detect *C. hominis* and *C. parvum* in one and five samples, respectively, which were mainly samples with low numbers of *C. parvum*/*C. hominis* reads (109–535 reads) by NGS. The qPCR assay has been tested extensively on human faecal samples and has been shown to have an analytical sensitivity of 1 oocyst/μL of DNA extract (Yang et al., 2013). This is the first time we have applied the qPCR to WWTP samples and no inhibition was observed.

In NSW, the dominant species detected were *C. hominis* and rat genotype III, whereas in QLD, *C. galli*, *C. muris* and *C. parvum* were the three most prevalent species, while in WA, *C. meleagridis* was the most prevalent species. Of these, *C. parvum* and *C. hominis* are the most common species reported in humans in Australia, accounting for >95% of human infections, with *C. meleagridis* the third most common species reported and usually accounting for 1–2% of notifications (Ryan and Power, 2012; Ng-Hublin et al., 2017). There have been numerous reports of *C. muris* in humans in other countries (cf. Ryan et al., 2017b). Other *Cryptosporidium* species with zoonotic potential, which were detected at a low prevalence in WWTP samples in the present study included *C. bovis*, *C. canis*, *C. cuniculus*, *C. erinacei*, *C. felis*, *C. scrofarum*, *C. suis* and *C. ubiquitum*. Nevertheless, caution is required when extrapolating any molecular data from WWTP samples to determine host sources of wastewater contamination by *Cryptosporidium*, as there are many potential input sources other than humans into wastewater networks. Unlike faecal material, there is no direct relationship between *Cryptosporidium* oocysts from wastewater samples and any potential host species, and an understanding of existing host-parasite interactions, parasite epidemiology and sources of faecal inputs into the wastewater network is required (Castro-Hermida et al., 2008).

A number of studies across the world have reported *C. hominis* (the predominant species in humans) among the most prevalent species detected in wastewater; Australia (King et al., 2015), Brazil and Peru (Ulloa-Stanojlović et al., 2016), China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017), Japan (Hashimoto et al., 2006; Hirata and Hashimoto, 2006), Switzerland and Germany (Ward et al., 2002), the USA (Xiao et al., 2001; Zhou et al., 2003) and Tunisia (Ben Ayed et al., 2012). In addition to anthroponotic sources of *C. hominis*, several studies in Australia have previously identified *C. hominis* in Australian cattle and wildlife including bandicoots (*Isoodon obesulus*), brushtail possums (*Trichosurus vulpecula*), eastern grey kangaroos (*Macropus giganteus*) and brush-tailed rock-wallabies (*Petrogale penicillata*) (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2016b; Zahedi et al., 2018). To date there is no conclusive molecular or epidemiological evidence linking contamination of wastewater by animals with the occurrence of *C. hominis* in raw wastewater or in human populations in Australia and further research is required in this area. In the present study, *C. hominis* was detected in NSW in plants C and D which received mainly septic tank waste and accounted for 93.0% and 77.3% of all *Cryptosporidium* species detected in plants C and D respectively, suggesting humans were the source. In NSW, *C. hominis* was detected in plants E and G. Plant E received a significant portion of trade waste and the *C. hominis* detected accounted for only 4.4% of all *Cryptosporidium* species identified, while plant G received mostly human waste and the *C. hominis* detected accounted for 26.6% all *Cryptosporidium* species identified.

In Europe, several studies have reported that *C. parvum* is the dominant species in wastewater (Hanninen et al., 2005; Spanakos et al., 2015; Imre et al., 2017; Ramo et al., 2017), while some studies in China, Iran, Tunisia and the USA have reported that livestock associated species such as *C. andersoni* and *C. xiaoi* dominate (Xiao et al., 2001; Liu et al., 2011; Ben Ayed et al., 2012; Hatam-Nahavandi et al., 2016). In the present study, *C. andersoni* and *C. xiaoi* were not detected in WWTPs across three states in Australia, however *C. parvum* was the third most prevalent species identified in QLD samples and was detected in a single sample and three samples from NSW and WA, respectively. *Cryptosporidium parvum* has been identified widely in both calves and humans in Australia (Ryan and Power, 2012) with reported prevalences for *C. parvum* in humans in Australia ranging from ~24% in Victoria (Jex et al., 2007; Koehler et al., 2013) to 17–19.8% in WA (Morgan et al., 1998; Ng et al., 2010) and 46.8% in NSW (Waldron et al., 2009). There are no published reports on the prevalence of *C. parvum* in the human population in QLD, which is a knowledge gap that needs to be addressed.

Table 5
C. hominis and *C. parvum* detected by NGS and species specific qPCR in individual wastewater treatment plant (WWTP) samples positive for *Cryptosporidium* spp. across three states of Australia; NSW, QLD and WA.

State	Plant	Sample	NGS		*C. hominis* and *C. parvum* oocysts numbers determined by species specific qPCR (MGB probes)	
			No of reads assigned to *C. hominis*	No of reads assigned to *C. parvum*	*C. hominis* oocysts/L	*C. parvum* oocysts/L
QLD	Plant A	QLD-A7	0	0	ND	ND
		QLD-A24	0	0	ND	ND
	Plant B	QLD-B2	0	0	ND	ND
		QLD-B3	**0**	**109**	**ND**	**61**
	Plant C	**QLD-C2**	**0**	**779**	**ND**	**470**
		QLD-C3	**0**	**246**	**ND**	**117**
		QLD-C19	**0**	**910**	**ND**	**482**
		QLD-C25	**0**	**133**	**ND**	**50[a]**
	Plant D	**QLD-D104**	**0**	**1181**	**ND**	**448**
		QLD-D140	0	0	ND	ND
	Plant E	**QLD-E18**	**0**	**562**	**ND**	**82[a]**
		QLD-E34	0	0	ND	ND
		QLD-E76	**109**	**918**	**76[a]**	**361**
		QLD-E195	0	0	ND	ND
		QLD-E222	0	0	ND	ND
		QLD-E258	0	0	ND	ND
		QLD-E303	0	0	ND	ND
		QLD-E357	0	0	ND	ND
		QLD-E375	**0**	**265**	**ND**	**47**
		QLD-E393	0	0	ND	ND
	Plant F	QLD-F1	0	0	ND	ND
		QLD-F33	**0**	**140**	**ND**	**2834**
		QLD-F84	**0**	**126**	**ND**	**51**
		QLD-F130	**0**	**189**	**ND**	**32**
		QLD-F157	0	0	ND	ND
		QLD-F319	0	0	ND	ND
		QLD-F382	**0**	**140**	**ND**	**38[a]**
	Plant G	**QLD-G53**	**0**	**113**	**ND**	**292[a]**
		QLD-G115	**2382**	**4336**	**386**	**812**
		QLD-G304	**0**	**20,571**	**ND**	**954**
		QLD-G331	0	0	ND	ND
		QLD-G340	0	0	ND	ND
	Plant H	**QLD-H8**	**0**	**18,529**	**ND**	**6314**
		QLD-H179	**0**	**1638**	**ND**	**922**
		QLD-H197	**0**	**4674**	**ND**	**1528**
		QLD-H386	0	0	ND	ND
	Plant I	**QLD-I41**	**0**	**1873**	**ND**	**476**
	Plant J	QLD-J15	0	0	ND	ND
		QLD-J47	0	0	ND	ND
		QLD-J354	0	0	ND	ND
		QLD-J363	0	0	ND	ND
	Plant K	QLD-K71	0	0	ND	ND
		QLD-K119	0	0	ND	ND
		QLD-K281	0	0	ND	ND
		QLD-K380	0	0	ND	ND
		QLD-K389	0	0	ND	ND
	Plant M	QLD-M60	0	0	ND	ND
	Plant N	QLD-N35	0	0	ND	ND
	Plant O	**QLD-O54**	**0**	**991**	**ND**	**14**
	Plant P	QLD-P63	0	0	ND	ND
	Plant Q	QLD-Q11	0	0	ND	ND
NSW	Plant B	**NSW-B2**	**0**	**20,347**	**ND**	**1380**
	Plant C	**NSW-C20**	**9227**	**0**	**2998**	**ND**
	Plant D	**NSW-D21**	**3082**	**0**	**3294**	**ND**
WA	Plant A	WA-A5	0	0	ND	ND
		WA-A8	0	0	ND	ND
		WA-A13	0	0	ND	ND
		WA-A16	0	0	ND	ND
		WA-A24	0	0	ND	ND
		WA-A37	0	0	ND	ND
		WA-A40	0	0	ND	ND
		WA-A55	0	0	ND	ND
		WA-A65	**0**	**884**	**ND**	**92**
		WA-A66	**0**	**2563**	**ND**	**214**
		WA-A68	**0**	**535**	**ND**	**51[a]**
		WA-A78	0	0	ND	ND
		WA-A79	0	0	ND	ND
		WA-A80	0	0	ND	ND
		WA-A81	0	0	ND	ND
		WA-A82	0	0	ND	ND
		WA-A88	0	0	ND	ND

Table 5 (*continued*)

State	Plant	Sample	NGS		*C. hominis* and *C. parvum* oocysts numbers determined by species specific qPCR (MGB probes)	
			No of reads assigned to *C. hominis*	No of reads assigned to *C. parvum*	*C. hominis* oocysts/L	*C. parvum* oocysts/L
		WA-A91	0	0	ND	ND
	Plant B	WA-B2	0	0	ND	ND
		WA-B4	0	0	ND	ND
		WA-B12	0	0	ND	ND
		WA-B13	0	0	ND	ND
		WA-B14	0	0	ND	ND
		WA-B19	0	0	ND	ND
		WA-B28	0	0	ND	ND
		WA-B30	0	0	ND	ND
		WA-B41	0	0	ND	ND
		WA-B42	0	0	ND	ND
		WA-B45	0	0	ND	ND

ND = not detected.
WWTP samples in which NGS detected *C. parvum* and/or *C. hominis* are in bold.
[a] For these samples, the *C. hominis* and *C. parvum* species-specific qPCR assay failed and oocyst/L is reported based on 18S qPCR and the percentage of NGS reads attributed to *C. hominis* and *C. parvum*.

Considering that most WWTPs in Australia are well fenced-off and protected, with minimal animal access, the predominance of *C. parvum* in wastewater in QLD may indicate that human sewage was the source of *C. parvum* or that it came from a combination of anthroponotic contributions and industry waste from abattoirs. In many of the QLD plants, a significant proportion was "trade waste" some of which may have come from abattoirs, however it was not possible to obtain further information on the sources of the trade waste. In WA, *C. parvum* was detected in plant A, which received both human and abattoir waste. In NSW, the single WWTP (Plant B) that was positive for *C. parvum* received waste predominately from septic tanks, suggesting an anthroponotic source. It is also important to remember that previous studies that reported Australian prevalence data for *Cryptosporidium* were from clinical samples, which in many cases were dominated by samples from the major metropolitan areas. Based on the population sizes for at least some of the WWTPs in the present study, most of the "urban sites" are more likely to be regional centres, so may have a different pattern of *Cryptosporidium* prevalence and species composition compared with major urban centres.

Cryptosporidium meleagridis is a common parasite of humans in Australia (Ryan and Power, 2012) and also infects a wide range of birds (Zahedi et al., 2016a), with many overlapping *C. meleagridis* subtypes found in both birds and humans; suggesting both anthroponotic and zoonotic transmission (Silverlas et al., 2012). This is evidenced by the fact that *C. meleagridis* is commonly reported in wastewater worldwide (Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Feng et al., 2009; Li et al., 2012; Huang et al., 2017). In the present study, *C. meleagridis* was the most prevalent species detected in WWTP samples collected from WA and in many cases was the only species detected (Supplementary Table 1). However, it was not detected in NSW or QLD. Although a variety of bird species are commonly seen at WWTPs in Australia, particularly around lagoons and clarifiers (secondary and tertiary treatment), the raw sewage entries to most WWTPs are covered, and not exposed and accessible to birds and animals. Some of the *C. meleagridis* detected in WWTPs in WA could have been originated from humans, however, further investigation revealed that the raw influent samples were taken directly from the distribution chamber located just before the primary ponds, which was only covered with a layer of mesh, providing easy access to bird contamination. Alternatively, industrial sources of wastewater from poultry farms could also be a major contributor. The predominance of the bird-specific *C. galli* in WWTP samples from QLD also confirms the potential role birds may play in contamination of wastewater by *Cryptosporidium*, but currently data on the contribution of poultry farms to WWTP in both WA and QLD is lacking and is an important knowledge gap. To date, there has only been one report of *C. galli* in wastewater (Ramo et al., 2017), however, *C. baileyi*, another avian *Cryptosporidium* species, has been reported in several studies from China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017). It is possible that the high levels of *C. meleagridis* and *C. galli* detected in WA and QLD respectively, were due to contamination in our laboratory. However, this is unlikely as neither species were included as controls on the same Illumina MiSeq run and quality filtering removed all reads <100. The high number of *C. meleagridis* reads in WA (107 to 58,246 reads/sample) and *C. galli* reads in QLD (129 to 32,164 reads/sample) supports their validity. In addition, if it was due to gross contamination, then both species would be randomly distributed across all samples, with mixtures of both species in some samples.

Two emerging human-pathogenic *Cryptosporidium* species, including *C. ubiquitum* ($n = 2$) and *C. cuniculus* ($n = 1$), were also found in wastewater samples from QLD at a lower frequency and abundance than other major species. *Cryptosporidium cuniculus* is a common parasite of rabbits and has been reported in source water in South Australia (Swaffer et al., 2018) and linked to several sporadic human cases in Australia (Nolan et al., 2010, 2013; Sari et al., 2013 unpublished - KF279538; Koehler et al., 2014), the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010). To date there are no published reports of *C. cuniculus* detected in WWTP samples in Australia, however, it has been previously reported from WWTPs in Brazil, Peru and China (Li et al., 2012; Ulloa-Stanojlović et al., 2016). Mainly infecting small ruminants, *C. ubiquitum* has been identified in a broad range of hosts including humans and wildlife (in particular rodents) with a wide geographic distribution across the world (Zahedi et al., 2016a). It has also been frequently reported from source water, stormwater runoff, stream sediment and wastewater across the world (Xiao et al., 2000; Nolan et al., 2013; Li et al., 2014). In Australia, *C. ubiquitum* has not been detected in the studies conducted to type *Cryptosporidium* isolates from humans (Ryan and Power, 2012); however, it has been identified in source water in Australia (Swaffer et al., 2018). More recently, the identification of similar *C. ubiquitum* subtypes in humans and in wastewater samples from China, Tunisia and the USA strengthens the hypothesis that sheep and wild rodents are a source of *C. ubiquitum* transmission to humans through contamination of untreated drinking water (Zhou et al., 2003; Liu et al., 2011; Ben Ayed et al., 2012; Li et al., 2014; Huang et al., 2017).

In the present study *C. muris*, a predominantly a rodent species of *Cryptosporidium*, was sporadically identified in wastewater samples from NSW and WA and was the second most prevalent species detected in QLD. There have been numerous reports of *C. muris* in humans and wastewater (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003;

Feng et al., 2009; Ben Ayed et al., 2012; Ryan and Power, 2012; Li et al., 2014; Spanakos et al., 2015; Huang et al., 2017), suggesting both human contribution as well as faecal contamination by rodents in wastewater distribution systems. However, as the frequency of detection of *C. muris* in humans is low (1–3%) (Wang et al., 2012), rodents are the more likely source. The identification of other rodent *Cryptosporidium* genotypes (rat genotypes I–IV) across all states in the present study, also supports this hypothesis.

Factors that influence oocyst density in wastewater are the incidence of cryptosporidiosis in the community (i.e. number of infected humans and animals in the community served by the WWTP), the intensity of infection (oocyst shedding), the size of the community (population), seasonality and dilution by other waste entering the WWTP (Domenech et al., 2017; King et al., 2017). In the present study, oocyst numbers per litre of sewage across the three states were estimated and ranged from 70 to 18,055 oocysts/L (mean = 3426 oocysts/L). This is similar to a previous study of WWTPs across South Australia and Victoria, with oocyst densities ranging from 3 to 21,335 oocysts/L with a mean density of 2355 oocysts/L (King et al., 2017). It is difficult, however, to compare across different studies using different methodologies. Worldwide, mean densities of between 10 and >700 oocysts/L have been commonly reported (Ajonina et al., 2012; Tonani et al., 2013; Nasser, 2016; Xiao et al., 2018) with a mean of 60,000 oocysts/L reported in one study (Cantusio Neto et al., 2006). The somewhat higher number of oocysts detected in the present study compared to other studies may be due to the fact that the oocyst concentrations were determined directly from total DNA extracted from WWTP samples by qPCR (using ddPCR calibrated standards), which may have overestimated the oocyst concentration, as DNA from lysed (and therefore no longer viable) oocysts would also have been detected. Previous studies have purified oocysts from WWTP samples and counted intact oocysts using USEPA method 1623, however, recovery efficiencies from wastewater samples can be highly variable, ranging from 5.5 ± 1.3% to as high as 85% (Nasser, 2016). The DNA extraction efficiency in the present study is unknown.

Estimation of *Cryptosporidium* risk from wastewater requires an evaluation of the efficiency of oocyst removal and inactivation along the treatment process and the reduction in the levels of oocysts (and their infectivity) in final treated effluent compared with oocyst counts in raw sewage (Xiao et al., 2018). Guideline values have traditionally set $\log_{10}$ removal targets based on end-use application (King et al., 2017), but these guidelines still do not incorporate the potential for inactivation of oocysts throughout the treatment process. A limitation of the present study is that samples were only taken from influent raw wastewater, and oocyst numbers were not investigated across the treatment train including the final effluent. Another limitation is that the viability/infectivity of oocysts detected in WWTP samples was not analysed. A recent study developed an integrated assay to determine oocyst density and infectivity from a single-sample concentrate (King et al., 2017), which will allow for improved QMRA analysis, as only analysing total oocyst numbers in raw sewage could result in an overestimation or underestimation of the *Cryptosporidium* risk in treated water. Finally, in the present study, the weather on sampling days (and preceding days) was not taken into account in the study design and future studies should include this data to better understand the effects of storm water intrusion for all the plants studied.

Conclusions: The current study has demonstrated that *Cryptosporidium* is prevalent in the raw influent of wastewater treatment facilities across Australia. NGS was central to unravelling the large diversity of *Cryptosporidium* species and genotypes in these samples and revealed the potential contribution of livestock, wildlife and birds (in addition to humans), to wastewater contamination. While human waste is a major contributor to WWTPs, the data from the present study suggests that abattoirs and poultry processing plants etc., could also be major contributors to wastewater treatment facilities. NGS analysis of the vertebrate species contributing to the wastewater will also help with determining the origin of the *Cryptosporidium* species detected in wastewater samples, and clearly further research is required to better understand the sources of *Cryptosporidium* in Australian wastewater. Comparisons between the results of the present study with previous studies which used Sanger sequencing are difficult, but as NGS becomes more widely used as the method of choice for typing pathogens in wastewater in the future, comparisons will become much more relevant and meaningful across studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.07.024.

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